

Melanoma antigen recognition by tumour-infiltrating T lymphocytes (TIL): effect of differential expression of Melan-A/MART-1

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SUMMARY

We have isolated, from an individual patient with metastatic melanoma, a series of eight TIL clones capable of lysing autologous melanoma cell targets. Six of the eight clones expressed TCRAV2S1 and lysed targets expressing HLA-A2 and the Melan-A/MART-1 peptide: AAGIGILTV. Polymerase chain reaction-single stranded conformational polymorphism (PCR-SSCP) analysis showed that the Melan-A/MART-1-specific clones were predominant in the bulk culture prior to cloning. However, the tumour progressed *in vivo* even in the presence of these tumour cell-lytic clones. Using the anti-Melan-A/MART-1 MoAb (A-103), we noted that Melan-A/MART-1 expression on three melanoma cell lines varied considerably during *in vitro* culture, in the absence of T cell immunoselection, relative to cell density. Tumour cells which spontaneously decreased Melan-A/MART-1 expression were less susceptible to specific TIL lysis. Melan-A/MART-1 expression and susceptibility to lysis increased in cells cultured at lower density. These data suggest that modulation of tumour antigen may account for tumour progression in the presence of tumour cell-lytic T lymphocytes. The observations suggest a possible explanation for the common finding of Melan-A/MART-1-specific lytic TIL in clinically progressing melanomas, as well as a possible pathway for therapeutic intervention.

Keywords tumour-infiltrating lymphocytes melanoma T cell receptor
cytotoxic T lymphocytes PCR-SSCP Melan-A/MART-1

INTRODUCTION

Several recent observations have demonstrated that spontaneously occurring human tumours display distinctive antigens which can be recognized by autologous lymphocytes [1–6]. In addition to the identification of several tumour antigens [7–9], immunodominant epitopes recognized by TIL have been described for widely expressed lineage-specific antigens, such as the HLA-A2-restricted Melan-A/MART-1 in melanomas [5,10]. Melanoma-associated antigens also include HLA-A2-restricted epitopes of antigens such as tyrosinase [11,12], gp100 [13,14], as well as a series of HLA-A1-restricted MAGE antigens [7]. We recently demonstrated that melanoma TIL can be activated, induced to proliferate, circulate and accumulate at the sites of tumours [15]. Such TIL would seem to fulfil the prerequisites for a successful cellular immune response, yet tumour cell-lytic T cells are often isolated from progressing tumours.

Melan-A/MART-1 is a lineage-specific differentiation antigen expressed in normal melanocytes and most melanomas [5,10]. Numerous studies have indicated that the T cell response against this antigen is restricted by HLA-A2, and that TIL present in melanomas often show a selective response to this antigen, particularly towards the immunodominant peptide AAGIGILTV (amino acids 27–35) [16]. Peptide immunization protocols have attempted to take advantage of the responsiveness to this antigen, but reported results indicate that although enhancement of cytotoxic activity was obtained, tumour regression was not achieved [17,18].

In the current study we have examined a series of autologous melanoma-specific TIL clones which were found to be selectively reactive with this same immunodominant Melan-A/MART-1 peptide. As reported in a previous case [15], using the polymerase chain reaction-single stranded conformational polymorphism (PCR-SSCP) technique we have demonstrated the predominance of a clone, in the uncloned 'bulk' culture, which represented three of the eight clones isolated; this clone shows specificity for Melan-A/MART-1. Despite the accumulation of these tumour cell-lytic TIL, the patient's tumour continued to progress.

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Others have indicated that there may be differential recognition of cultured tumour cell lines and freshly isolated tumours [19], as well as selective loss of MART antigen *in vivo* [18,20,21]. We wished to determine if Melan-A/MART-1 antigen is modulated *in vitro* during tumour cell culture, and if this modulation is correlated with the ability to lyse the targets *in vitro*. Our results on three Melan-A/MART-1⁺ melanomas indicate that tumours show diminished Melan-A/MART-1 expression when they are cultured *in vitro* at high cell density. Melan-A/MART-1 protein expression could be restored when tumour cell lines were returned to low density. These data suggest that Melan-A/MART-1 antigen expression, and the ability to recognize tumour cells, can be modulated *in vitro*. Unlike previous reports, which have emphasized the selection of Melan-A/MART-1⁺ tumours in the face of a T cell immune response [20–24], these data demonstrate that tumours can regulate their own antigen expression in the absence of any lymphocytes. This observation may provide a model for study of regulation of tumour antigen expression, and the concomitant cell-mediated immune response against tumours.

MATERIALS AND METHODS

Isolation of TIL and tumour

Specimens were isolated from biopsies of metastatic malignant melanomas according to approved institutional guidelines at the Massachusetts General Hospital. TIL were propagated in recombinant IL-2 as previously described [25–27]. When sufficient TIL were available, they were tested for cytotoxic activity against tumour cells, phenotyped by flow cytometry, and processed for PCR amplification as described below.

T cell cloning

TIL were cloned by limiting dilution using irradiated mononuclear feeder cells together with phytohaemagglutinin (PHA) as a polyclonal stimulus as previously described [2,28,29]. Limiting dilution was performed on TIL which had been in culture for 2 weeks prior to cloning. Clones were expanded in IL-2; a minimum of 5×10^7 cells were utilized for functional assays and PCR analyses.

Autologous tumour cell lines

The tumour cell lines used in this study (MU, MO and MA) have been previously described [2,30]. Tumour cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% human serum (MU), or in RPMI 1640 supplemented with 5% human serum (MA and MO). The melanoma origin of the lines was confirmed using antibodies to melanoma-associated antigen S-100 and HMB-45 [31]. Both MU and MA tumours expressed HLA-A2; MU tumour cells were also HLA-A1⁺. MO tumour was derived from a patient whose leucocytes expressed HLA-A2, while MO tumour cells did not express this class I MHC antigen.

Functional analyses

Screening of the cytotoxic activity of TIL bulk and cloned progeny was determined against autologous tumour (MU), allogeneic melanomas, as well as natural killer (NK) (K562) and LAK (Daudi), and Epstein–Barr virus (EBV)-transformed B lymphocyte targets: EBV-3 (HLA-A1, B8, DR3), EBV-19 (HLA-A2, B18, DR5), using a ⁵¹Cr-release assay, as previously described [25]. Clones were screened for cytotoxic activity at effector to target ratios of 50:1 and below.

Peptide-specific lysis of targets

To test for the fine specificity of melanoma cell-lytic clones we incubated an HLA-A2⁺ lymphoblastoid (EBV-19, see above), with the following melanocyte lineage-derived peptides:

Melan-A/MART-1 [10]:	AAGIGILTV
Tyrosinase [11]:	MLLAVLYCL
	YMNGTMSQV
MAGE-3 [32]:	EVDPIGHLY

Peptides were added to the target cells at 5 µg/ml for 2 h prior to labelling with ⁵¹Cr in order to allow surface expression of peptide–HLA complexes. The peptide-pulsed targets were then used in a ⁵¹Cr-release assay as above.

Expression of Melan-A/MART-1 by tumour cells

Expression of the melanocyte lineage-specific antigen Melan-A/MART-1 was assessed by staining with MoAb A-103 shown to stain cytoplasmic Melan-A/MART-1 protein [33]. Following fixation in 1% paraformaldehyde, cells were semipermeabilized using 0.1% saponin for 5 min to allow entry of antibodies into the cytoplasm. Aliquots of 2×10^5 tumour cells were stained at room temperature for 45 min with the mouse anti-human Melan-A/MART-1 MoAb A-103, following two washes in 0.1% saponin/PBS, and a second stain with FITC-conjugated goat anti-mouse immunoglobulin for 30 min at room temperature. Intensity of Melan-A/MART-1 staining is expressed as mean channel of fluorescence of all gated cells using a Becton Dickinson FACScan.

Analysis of TCR usage

TCR usage was assessed for propagated bulk TIL and TIL clones, using a PCR amplification system for TCR α and β genes as previously described [34]. Total RNA was isolated from 10^7 actively growing cells using RNeasy (Qiagen/Biotech International Inc., Friendswood, TX) as described [35]. PCR products from appropriate V-region transcripts of TIL clones were sequenced by the Sanger dideoxy chain termination method [36] using the Sequenase Kit (Version 2; United States Biochemical Corp., Cleveland, OH) as described by the manufacturer.

SSCP analysis

PAGE by SSCP [37,38] was adapted for the analysis of the TCRAV2S1 TIL clones obtained in this study. In order to obtain a PCR product of suitable size for SSCP analysis [38], the primary PCR product (approximately 800 bp) was reamplified using internal 5' TCRAV2 and 3' TCRAC primers to produce a nested PCR product of 200 base pairs spanning the TCRAV-N-J joint. The primers employed were as follows: Outer primer pair, TCRAV2S1 5'gtgttcagaggagccattgcc3', TCRAC 5'cctagatgggcttaatacga3'; nested primer pair, TCRAV2 5'gccagtatgttctctgctca3', TCRAC 5'acagactgtcactggattagag3'. SSCP analysis and extraction of DNA fragments for sequencing were performed as previously described [15].

RESULTS

Phenotype and function of TIL bulk and clones

The IL-2 responding TIL propagated from patient MU were over 98% CD3⁺, CD8⁺ (CD4[−]) T cells expressing the $\alpha\beta$ TCR. As previously reported [2], strong cytotoxic activity was noted, when tested in a ⁵¹Cr-release assay against the autologous MU-melanoma tumour cell line. This bulk TIL line showed minimal NK or LAK activity, as it was only weakly lytic to K562 or Daudi

Table 1. Summary of functional activities and TCR usage by MU-TIL clones

TIL clone	Auto-tumour killing*	K562 (NK) killing*	TCR α chain	TCR β chain	Comment
MU-45	+++	–	V2S1J41	V19D1J2S6	Three clones have identical TCR
MU-63	+++	–	V2S1J41	V19D1J2S6	
MU-79	++++	–	V2S1J41	V19D1J2S6	
MU-9	+++++	–	V2S1J35	V14D2J2S7	Two clones have identical TCR
MU-115	+++	–	V2S1J35	V14D2J2S7	
MU57	+++++	–	V2S1J22	V5S8D1J1S1	TCR α chain similar to 8B3 [43]
MU-10	++++	–	V2S5J27 V23J10	V3D2J2S3	Two clones have identical TCR β chains, but different TCRAJ and additional different TCRAV
MU-58	+++	–	V2S5J23 V15J15	V3D2J2S3	
MU-135	–	++++	V7S2J27	V6D1J2S2	NK killing only, no melanoma killing

*Tumour target lysis is shown as follows: –, <10% specific lysis; +, 10–20%; ++, 20–40%; +++, 40–60%; +++++, 60–80%; ++++++, >80%.

target cells [2,30]. The ability of bulk MU-TIL to lyse autologous tumour targets was blocked by antibodies to HLA class I antigens (W6/32). Furthermore, the TIL did not lyse the EBV-transformed B cell lines, EBV-3 or EBV-19.

From limiting dilution of the bulk culture (MU-TIL), a total of 145 MU-TIL clones were isolated as previously described [2]. As summarized in Table 1, for further analysis we selected eight of the clones which showed consistent anti-tumour cytotoxicity (at <25:1 effector to target ratio), without NK activity.

TCR usage by MU-TIL

Bulk MU-TIL. Bulk MU-TIL and MU-TIL clones were analysed for TCR-V-gene usage using a panel of family-specific oligonucleotide primers. These reverse transcriptase (RT)-PCR analyses revealed that TCRAV-2, 13, 17 and 23 represented >10% of the total TCRAV transcripts among these bulk MU-TIL (see Fig. 1a). Several other TCRAV genes were represented to a lesser degree, and several TCRAV genes were not detected at all. RT-PCR analysis to evaluate usage of TCRBV genes by bulk MU-TIL revealed that TCRBV-6, 7, 14 and 19 represented \geq 10% of the total TCRBV transcripts. Several of the TCRBV transcripts were virtually undetectable (see Fig. 1b).

MU-TIL clones. Table 1 summarizes the TCR usage by the nine cytotoxic TIL clones from melanoma patient MU (eight melanoma cell-lytic clones, and a single NK-lytic clone). Nucleotide (and derived amino acid) sequences of the CDR3 of the TCR α chains of the anti-melanoma-specific clones are shown in Fig. 2a, and the CDR3 sequences of the TCR β chains are shown in Fig. 2b. The eight cytotoxic clones (*versus* autologous tumour cells) displayed a distinct non-random pattern of TCRAV gene usage. Of the eight melanoma cell-lytic clones, six transcribed TCRAV2S1, and the other two melanoma cell-lytic clones transcribed TCRAV2S5 (MU-10 and MU-58). TCRBV usage was somewhat more diverse than the TCRAV usage. Three of the TCRAV2S1 (clones MU-45, 63 and 79) had identical nucleotide sequences for the TCR α

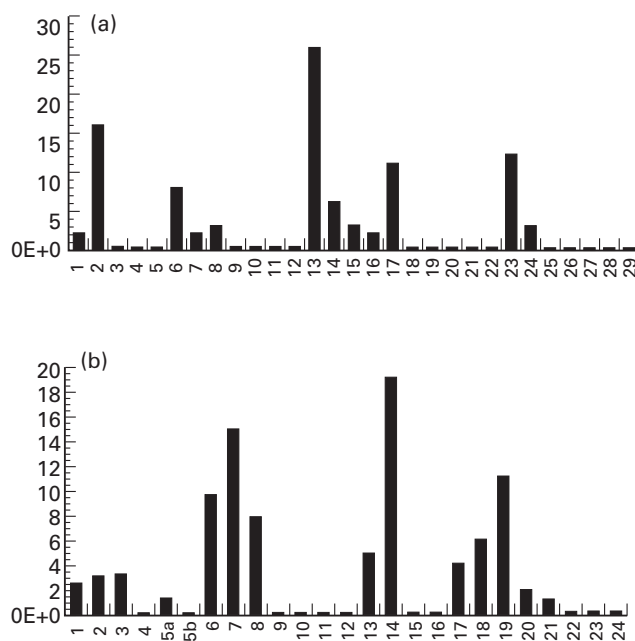


Fig. 1. Comparison of TCRV gene usage by MU-TIL. (a) Polymerase chain reaction (PCR) analysis of MU-TIL TCRAV gene usage. A panel of family-specific oligonucleotide primers specific for 29 different TCRAV genes was used for PCR amplification of cDNA transcribed from extracted RNA from the 'bulk' MU-TIL culture. The percentage usage for each TCRAV gene family was calculated as a percentage of the total of TCRAV. (b) PCR analysis of MU-TIL TCRBV gene usage. A panel of family-specific oligonucleotide primers specific for 25 different TCRBV regions genes was used for PCR amplification of cDNA transcribed from extracted RNA from the 'bulk' MU-TIL culture. The percentage usage for each TCRBV gene family was calculated as a percentage of the total of TCRBV.

(a)

TCR-ALPHA CHAIN NUCLEOTIDE SEQUENCES

		V-REGION	CDR3-REGION
MU-9,115	TCRAV2S1J35	TGTGCCGCTTCA	TTAGGCTTTGGGAATGTGCTGCAT
MU-45,63,79	TCRAV2S1J41	TGTGCCCTTAAA	GGGAGGTCAAATTCGGGTATGCACTCAACTTCGGCAAAGGC
MU-10	TCRAV23J10	TGTGCTGTGGAG	CGGGTCACGGGGAGGAGGAAACAACCTCACCTTTGGGACAGGC
MU-10	TCRAV2S5J27	TGTGTGCTCCCG	GGGGCAATGCAGGCAAATCAACC
MU-58	TCRAV2S5J23	TGTGTGCTGGTT	TATAACCAGGGAGGAAAGCTTATC
MU-58	TCRAV15J9S5	TGTGCTTTCAAC	CAGGCAGGAAGTCTCTGATC
MU-57	TCRAV2S1J22	TGTGCCGTGAAC	AGTGTTCTGCAAGGCAACTGACC
MU-135	TCRAV7S2J27	TGTGCTGCTAAC	ACCAATGCAGGCAAATCAACC

TCR-ALPHA CHAIN DERIVED AMINO ACID SEQUENCES

		V-REGION	CDR3-REGION
MU-9,115	TCRAV2S1J35	C A A S L G F G N V L H	C G S G
MU-45,63,79	TCRAV2S1J41	C A L K G R S N S G Y A L N F G K G	
MU-10	TCRAV23J10	C A V E R V T G G G N K L T F G T G	
MU-10	TCRAV2S5J27	C V V P G A N A G K S T	F G D G
MU-58	TCRAV2S5J23	C V V V Y N Q G G K L I	F G Q G
MU-58	TCRAV15J9S5	C A S N Q A G T A L I	F G K G
MU-57	TCRAV2S1J22	C A V N S G S A R Q L T	F G S G
MU-135	TCRAV7S2J27	C A A N T N A G K S T	F G D G

(b)

TCR-BETA CHAIN NUCLEOTIDE SEQUENCES

		V-REGION	CDR3-REGION
MU-9,115	TCRBV14D2J2S7	TGTGCCAGCAGA	ACCCCCGGCGGCCACGAGCAGTAC
MU-45,63,79	TCRBV19D1J2S6	TGTGCCAGTACC	TGGGGGGCCCTTTTCGGCCAACGTCCTGACT
MU-10,58	TCRBV3D2J2S3	TGTGCCAGCAGT	TTTGGTTCGGATCCTCACAGATACGCAGTAT
MU-57	TCRBV5bD1J1S1	TGTGCCAGCAGC	TTAGCAGGGTTGGGCACTGAAGCTTTC
MU-135	TCRBV6D1J2S2	TGTGCCAGCAGA	TATGTACAGGGACCCGGGGAGCTGTTT

TCR-BETA CHAIN DERIVED AMINO ACID SEQUENCES

		V-REGION	CDR3-REGION
MU-9,115	TCRBV14D2J2S7	C A S R T P G G H E Q Y	F G P G
MU-45,63,79	TCRBV19D1J2S6	C A S T W G A L S A N V L T F G A G	
MU-10,58	TCRBV3D2J2S3	C A S S F G R I L T D T Q Y F G P G	
MU-57	TCRBV5bD1J1S1	C A S S I R N L G T E A F	F G Q G
MU-135	TCRBV6D1J2S2	C A S R Y V Q G P G E L F	F G E G

Fig. 2. TCR sequences of TIL clones. (a) TCR α chain sequences of CDR3 of MU-TIL clones. TCR α chain nucleotide and derived amino acid sequences of the eight TIL clones with autologous melanoma-specific cytotoxic activity. (These sequence data are available from EMBL/GenBank/DBJ under accession numbers U41470, U40463, U41470.) (b) TCR β chain sequences of CDR3 of MU-TIL clones. TCR β chain nucleotide and derived amino acid sequences of the CDR3 of eight TIL clones with autologous melanoma-specific cytotoxic activity. (These sequence data are available from EMBL/GenBank/DBJ under accession numbers U40815, U41097, U40831.)

chain (TCRAV2S1J41) and TCR β chain (TCRBV19D1S1J2S6) transcripts. Likewise, two other clones, MU9 and 115, were identical in TCR usage (TCRAV2S1J35, TCRBV14D2S1J2S7). The other TCRAV2S1 clone, MU-57, used TCRAJ22 and TCRBV5S8D1S1J1S1.

The remaining two melanoma cytotoxic clones, MU10 and 58, showed identical nucleotide sequences of their TCRBV3 transcripts (TCRBV3D2J2S3) (Fig. 2b). However, two different, in-frame TCR α chain transcripts were detectable in each clone. Although TCRAV2S5 was detected in both clones, they transcribed different TCRAJ (MU-10 = TCRAV2S5J27; MU-58 = TCRAV2S5J23). In addition, MU-10 expressed a second, in-frame TCRAV23J10, while MU-58 transcribed TCRAV15J15 (Fig. 2a). Thus, by PCR analysis, MU-10 and MU-58 shared identical TCR β chain transcripts at the nucleotide level, although each possessed two different TCR α chain transcripts.

SSCP analysis of TCRA2S1 transcripts

Figure 3 shows the TCRAV2S1 SSCP 'fingerprint' of bulk MU-TIL culture and the six TCRAV2S1 clones isolated from this bulk culture. In lane 1, the SSCP pattern of the bulk culture revealed two dominant bands (arrows) which migrated to the same position on the gel as the bands from clones MU-45, -63 and -79 (lanes 3–5). Clones MU-9 and -115 share a different banding pattern, and clone MU-57 yet another banding pattern. In order to confirm whether the banding pattern in the bulk culture and clones MU-45, -63 and -79 were reflective of the abundant representation of this clone in the bulk population, the designated bands from lane 1 were cut, eluted, reamplified, and sequenced. The sequence obtained matched perfectly with the TCR α chain sequences of MU-45, -63, and -79 lymphocyte clones. These analyses thus revealed that these clones were present at elevated levels in the bulk culture.

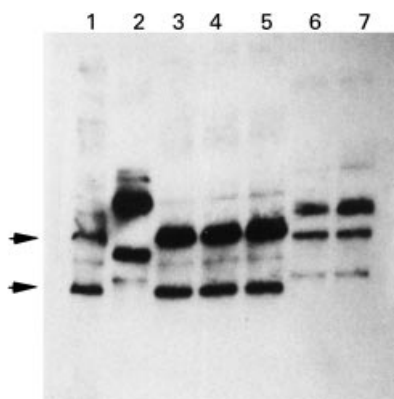


Fig. 3. Polymerase chain reaction-single stranded conformational polymorphism (PCR-SSCP) analysis of TCRAV2S1 TIL clones and bulk TIL. PCR-amplified TCRAV2S1 transcripts of MU-TIL were analysed by SSCP. Six clones, including MU-9 (lane 7), MU-115 (lane 6), MU-45 (lane 5), MU-63 (lane 4), MU-79 (lane 3), and MU-57 (lane 2), which all use TCRAV2S1, are compared with the TCRAV2 transcript from the bulk culture (lane 7) from which these clones were isolated. The strong upper band in the bulk culture (arrow) was extracted and the DNA sequenced to show that it is identical to the TCR α chain sequence of clones MU-45, -63 and -79 which show a band with similar migration.

Peptide specificity of MU-TIL

The bulk MU culture and clones MU-45, MU-57, MU-63, MU-79 and MU-115 showed strong reactivity against EBV-19 targets pulsed with Melan-A/MART-1 peptide (AAGIGILTV) (not shown). Two additional clones (MU-10 and MU-58) failed to lyse these EBV targets pulsed with the Melan-A/MART-1. None of the T cells lysed the EBV targets alone, or these targets pulsed with tyrosinase or MAGE-3 peptides (not shown). One of the clones, MU-9, which lysed autologous melanoma targets could not be recovered from the freezer for testing for fine specificity with the melanoma peptides, although its TCR was identical to MU-115, indicating that MU-9 was also likely to be Melan-A/MART-1 peptide-specific. As noted, clones MU-45, MU-63 and MU-79 were identical to one another as determined by TCR gene sequencing.

Modulation of Melan-A/MART-1 on melanoma cell lines

We noted that the ability of the bulk MU-TIL and MU-TIL clones to lyse MU tumour targets varied considerably over time; particularly when tumour cells were grown for protracted periods of time in the same culture vessel without trypsinization of strongly adherent cells. The ability of the TIL to lyse such tumour targets was notably diminished. We therefore tested the tumour cells for the presence of both the Melan-A/MART-1 antigen (the target antigen for six of the clones), and HLA class I antigen required for peptide antigen presentation.

As shown in Figs 4 and 5, the intensity of intracytoplasmic Melan-A/MART-1 antigen expression in tumour cells varied widely, depending on *in vitro* culture conditions. The ability of TIL to lyse the tumour cells was diminished in direct proportion to the intensity of cytoplasmic Melan-A/MART-1 expression (Fig. 5).

As shown in Fig. 4a, when the MU tumour cells were cultured at high density (5×10^5 cells/ml) the mean channel fluorescence of Melan-A/MART-1 was approximately half that of the same tumour cells cultured at 1×10^5 cells/ml. As shown in Fig. 5a, the susceptibility to lysis of MU tumour cells by TIL was similarly reduced by approximately half on the low expressors (cultured at higher cell density) in comparison with the high expressors (cultured at lower cell density). Further evidence that the diminished cytotoxicity noted with these high-density tumour cells was related to Melan-A/MART-1 expression is the finding that addition of Melan-A/MART-1 peptide to the low-expressor tumour targets, MU-Lo and MU-X, restored high levels of cytotoxicity (Fig. 5b). The specificity of TIL in this assay is further demonstrated by their ability to lyse HLA-A2-expressing EBV-transformed B cells only after they were pulsed with Melan-A/MART-1 peptide (Fig. 5b).

Diminished Melan-A/MART-1 expression (Fig. 4b) paralleled decreased target cell lysis for a second tumour cell (MA), which expressed both Melan-A/MART-1 and HLA-A2 (not shown). A third melanoma tumour target (MO) also showed diminished Melan-A/MART-1 expression with increasing culture density (Fig. 4c), although the absence of HLA-A2 on this tumour rendered it non-susceptible to lysis by the TIL (not shown).

As shown in Fig. 4d, while Melan-A/MART-1 decreased among cells cultured at high density, the surface staining of HLA class I antigen did not change significantly under the same

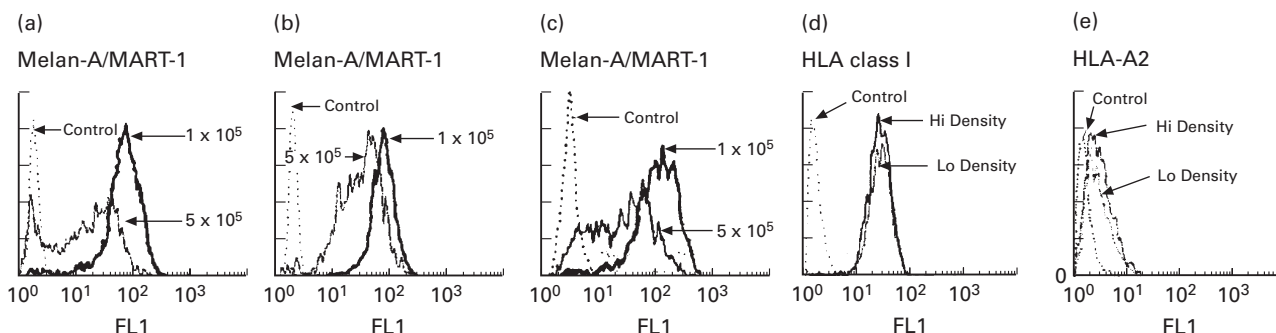


Fig. 4. Melan-A/MART-1 expression: modulation with culture density. Three different melanoma tumour lines were stained for cytoplasmic Melan-A/MART-1 with antibody A-103 (a–c) or class I HLA antigen (W6/32) (d). Each panel compares Melan-A/MART-1 expression on cells cultured at different densities (1×10^5 cells/ml versus 5×10^5 cells/ml). (a) Tumour MU stained with Melan-A/MART-1. (b) Tumour MA stained with Melan-A/MART-1. (c) Tumour MO stained with Melan-A/MART-1. (d) Tumour MU stained with W6/32. (e) Tumour MU stained with anti-HLA-A2.

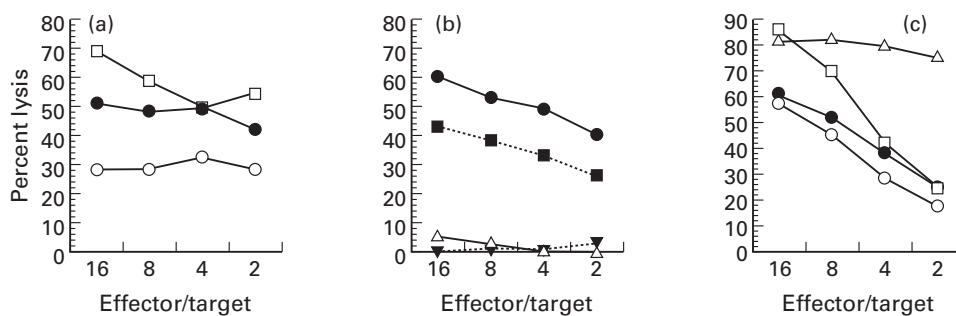


Fig. 5. Lysis of tumour targets: effect of MART-1/Melan-A expression on target cell recognition. (a) MU-TIL lysis of autologous MU tumour. MU tumour with high constitutive Melan-A/MART-1 expression (●; mean channel FL = 182) is more susceptible to lysis by MU-TIL than MU tumour cultured at high density, with lower Melan-A/MART-1 expression (○, MU-Lo; mean FL = 80). Pulsing of MU-Lo with Melan-A/MART-1 peptide (□) enhances susceptibility to lysis. (b) MU-TIL lysis of melan-A/MART-1⁻ targets. MU tumour selected for deficient expression of Melan-A/MART-1 (MU-X, ▼) is not lysed by MU-TIL, nor is an HLA-A2-expressing Epstein-Barr virus (EBV) cell line (△). Both targets EBV + Pep (●) and MU-X + Pep (■) can be lysed if they are pulsed with Melan-A/MART-1 peptide. (c) Anti-HLA-A2-specific lysis of targets. The MU tumour variants MU-Hi (○), MU-Lo (●) and MU-X (□) as well as the HLA-A2-expressing EBV cell line (△) can be lysed by an allo-specific anti-HLA-A2-directed cytotoxic T cell line, indicating that this restricting element is present on all of the target cells.

conditions. The HLA-A2 staining, although weaker than that noted with the broad specificity HLA class I antibody, also did not change with culture conditions (Fig. 4e), indicating that this restricting element was not the limiting factor in the diminished lytic susceptibility. The unaltered HLA staining also indicates that not all proteins were decreased in parallel with Melan-A/MART-1 when the tumour cells were cultured at higher cell density. Furthermore, the continued presence of HLA-A2, and its ability to serve as a target antigen for cytotoxic T cell recognition, are reflected by the ability of HLA-A2-specific alloreactive T cells to lyse the low HLA-A2-expressing tumour cells (Fig. 5c), as well as a Melan-A/MART-1-deficient variant of the MU tumour, although the Melan-A/MART-1-specific T cells showed diminished target recognition with reduced Melan-A/MART-1 expression (see Fig. 5a).

The decrease in Melan-A/MART-1, in cells cultured at higher density, was reversible when the cells were passaged at lower density. Cells cultured at high density showed diminished Melan-A/MART-1 expression (mean channel fluorescence 48.9). If these cells were passaged at high density (5×10^5 cells/ml), they continued to show low Melan-A/MART-1 (mean channel 40.9), while culture of the same cells for 7 days beginning at 1×10^5 cells/ml resulted in an approximate doubling of Melan-A/MART-1 expression (mean channel 108.9). Successive passage of cells at 1×10^5 cells/ml allowed higher retention of higher levels of Melan-A/MART-1 expression (mean channel 112.1), but when these cells were allowed to grow to high density over a subsequent 7-day culture period, the resulting culture again showed decreased Melan-A/MART-1 intensity (mean channel 46.9). These results indicate the cycling of Melan-A/MART-1 expression in relation to the time in culture and cell density. As noted in Fig. 5, the decrease in Melan-A/MART-1 intensity was paralleled with a decreased susceptibility to lysis.

DISCUSSION

This study demonstrates the isolation of Melan-A/MART-1-specific cytotoxic TIL from progressing tumours. The results also demonstrate the ability of melanoma tumour cells to modulate expression of this immunodominant antigen *in vitro* in the absence

of T cell immunoselection. These data indicate that there may be strong antigen expression on some tumour cells, allowing for recruitment of specific T lymphocytes, while other tumour cells have diminished antigen expression, and thus diminished susceptibility to T cell recognition and lysis. These findings could explain the simultaneous infiltration with antigen-specific cells and the development of antigen-negative tumour populations which can escape immune destruction.

Our TCR analyses indicate that although bulk MU-TIL express multiple TCRAV and TCRBV gene transcripts, the tumour cell-lytic clones consistently utilized TCRAV2 transcripts. The finding that bulk TIL and the TCRAV2S1-expressing TIL clones were all reactive with the same Melan-A/MART-1 peptide demonstrates the strong selective pressure for reactivity to this HLA-A2-restricted antigen, confirming the observations of Kawakami *et al.* [10] and Sensi *et al.* [5]. Previous studies on TCR restriction in melanoma TIL have shown differing results, with some groups failing to detect TCR restrictions [39,40], and others detecting restricted TCR repertoires [4,41]. Our own study of the bulk MU-TIL indicates a possible explanation for these discordant conclusions, as the overall TCR usage may be much more diverse than the antigen-specific component. For example, we observed a high frequency of the TCRAV13 in the PCR studies of the bulk MU-TIL, but this TCRAV was not reflected within the tumour cell-lytic clones we studied. It has recently been noted that the T cells infiltrating regressing tumours have different TCRBV usage than those in areas where tumours are progressing, in the same biopsy, further emphasizing the selection which occurs at the site of antigenic stimulation [42].

Sharing of TCR elements by tumour cell-lytic TIL in our studies correlates with previously reported findings, particularly with respect to Melan-A/MART-1-specific clones, including the combination of TCRAV2S1 with TCRBV14 reported by Sensi *et al.* [5], and seen in two of our clones. Our clone MU-57 differs from the Sensi clone, 8B3 [43], by only two amino acids in the CDR3. 8B3 has lost the germ-line codon for asparagine which is retained by MU-57, but has added codons for tyrosine and an adjacent histidine. Although the TCRAV2S5-expressing clones, MU-10 and MU-58, did not react with these Melan-A/MART-1 peptides, or with another HLA-A2-restricted peptide derived from

tyrosinase, the TCRBV3-D2S1-BJ2S3 used by these clones was also predominant among melanoma TIL noted by Puisieux *et al.* [41]. However, extensive N-region diversity, as well as use of different portions of the TCRBD, yielded significantly different CDR3 amino acid sequences for these TCR.

In addition to the TCR sequence analyses, the PCR-SSCP analysis provides evidence for the selection of anti-tumour T cells among the bulk TIL. These results parallel our previous report in another patient [15], in which SSCP demonstrated the presence of the TCR DNA sequence of an anti-tumour cell-lytic T cell clone in two different tumour deposits, and within the fresh tumour tissue prior to any culture, in part addressing the concern that the observed dominance in patient MU was simply the result of *in vitro* outgrowth of a subset of cells, rather than a reflection of the predominance *in vivo*.

Despite several lines of evidence indicating the ability of both normals and tumour patients to generate T lymphocytes specific for Melan-A/MART-1, and its peptide AAGIGILTV, the inability of peptide-immune individuals to eradicate the tumour stands in stark contrast to the lytic ability of T cells specific for tumours expressing this antigen [17,18]. Clearly, the escape of tumours from immune recognition can be the result of numerous changes in tumour cells and their antigen expression. As several laboratories have noted, there are tumour variants which no longer express the Melan-A/MART-1 antigen [18,21,44], or have lost the restricting HLA allele [23,30], or show defective antigen processing [20], all of which are needed for T cell recognition of the peptide antigen. It is noteworthy that in each of these reports, the role of the immune response in selecting antigen-negative variants, presumably mutants, has been emphasized. However, to the extent that Melan-A/MART-1 diminishes spontaneously, in the absence of T cells, as in our high-density tumour cultures, the natural modulation of this tumour antigen may explain not only a mechanism for tumour escape, but also could explain the continued presence of T cells at the sites of tumour deposits. Also, the fall in Melan-A/MART-1 expression we have noted appears to be independent of HLA class I expression, as class I MHC did not fall in cells which showed diminished Melan-A/MART-1.

In answer to the apparent dilemma that Melan-A/MART-1-specific TIL persist, but are ultimately ineffective at eradicating the tumour *in vivo*, the data we present, concerning *in vitro* modulation of Melan-A/MART-1 expression, present a possible explanation. If the tumour still has a significant proportion of cells expressing Melan-A/MART-1, then there will be a continued stimulus for lymphocyte infiltration, even if some of the cells continue to grow in the presence of the specific T cells because of their down-modulation of Melan-A/MART-1 expression. If, and when, the tumour permanently loses the ability to express Melan-A/MART-1 (or the restricting HLA-A2), either through down-regulation of gene expression or loss of genes through mutation, the lymphocytes would no longer have a stimulus for infiltration and accumulation; thus the tumour would continue to grow with the poor prognostic indicator of diminished lymphocyte numbers [45,46].

The observed modulation of Melan-A/MART-1 expression may not only correlate with immune responses to the tumour, but also presents a potential avenue for intervention if Melan-A/MART-1 expression could be maintained. If the regulatory elements for Melan-A/MART-1 expression can be elucidated, and its expression enhanced, it might be possible to generate a more effective immune response able to control melanoma tumour growth. Indeed, it is possible that spontaneous tumour regression,

and the rare successes observed in immunotherapy of melanoma patients, may reflect not only intensification of the T cell response, but also the concomitant constitutive expression of the target melanoma antigen(s).

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